

**HUMAN VASCULAR IBP-LIKE GROWTH FACTOR**

*1/15/91*  
This invention relates to newly identified polynucleotides, polypeptides encoded by such polynucleotides, the use of such polynucleotides and polypeptides, as well as the production of such polynucleotides and polypeptides. The invention also relates to inhibiting the action of such polypeptides.

The polypeptide of the present invention is related to a family of growth regulators comprising cef 10/cyr 61, connective tissue growth factor (CTGF), and nov, as well as the insulin-like growth factor binding protein (IBP) family which modulates the activity of insulin-like growth factor (IGF). The mRNA corresponding to the polypeptide of this invention is highly expressed in vascular cell-types, thus, this polypeptide is hereinafter referred to as human vascular IBP-like growth factor or "VIGF".

Growth factors and other mitogens, including transforming oncogenes, are capable of rapidly inducing a complex set of genes to be expressed by certain cells (Lau, L.F. and Nathans, D., Molecular Aspects of Cellular Regulation, 6:165-202 (1991). These genes, which have been named immediate early or early response genes, are transcriptionally activated within minutes after contact with a growth factor or mitogen, independent of de novo protein

synthesis. A group of these immediate early genes encodes secreted, extracellular proteins which are needed for coordination of complex biological processes such as differentiation and proliferation, regeneration and wound healing (Ryseck, R.P. et al, Cell Growth Differ., 2:235-233 (1991).

Highly related proteins which belong to this group include *cef 10* from chicken, which was detected after induction by the viral oncogene pp60<sup>src</sup> (Simmons, D.L. et al, PNAS, U.S.A., 86:1178-1182 (1989). A closely related protein, *cyr 61*, is rapidly activated by serum or platelet-derived growth factor (PDGF) (O'Brien, T.P. et al, Mol. Cell Biol., 10:3569-3577 (1990). The overall amino acid identity between *cef 10* and *cyr 61* is as high as 83%. A third member is human connective tissue growth factor (CTGF) (Bradham, D.M. et al., J. Cell. Biol., 114:1285-1294 (1991). CTGF is a cysteine-rich peptide which is secreted by human vascular endothelial cells in high levels after activation with transforming growth factor beta (TGF- $\beta$ ). CTGF exhibits PDGF-like biological and immunological activities and competes with PDGF for a particular cell surface receptor.

A fourth member of the immediate-early proteins is *fisp-12*, which has been shown to be induced by serum and has been mapped to a region of the murine genome (Ryseck, R.P. et al., Cell Growth Differ., 2:235-233 (1991). Yet another member of this family is the chicken gene, *nov*, normally arrested in adult kidney cells, which was found to be overexpressed in myeloblastosis-associated virus type 1 induced nephroblastomas. Further, expression of an amino-terminal-truncated *nov* product in chicken embryo fibroblasts was sufficient to induce transformation (Joliot, V. et al., Mol. Cell. Biol., 12:10-21 (1992).

The expression of these immediate early genes act as "third messengers" in the cascade of events triggered by growth factors. It is also thought that they are needed to

integrate and coordinate complex biological processes, such as differentiation and wound healing in which cell proliferation is a common event.

This emerging family of growth regulators is called the CCN family for CTGF; cef 10/cyr 61; and nov. The VIGF polypeptide of the present invention is thought to be a member of this family of growth regulators. The VIGF polypeptide also contains a stretch of cysteines which is highly homologous to insulin-like growth factor (IGF)-binding protein.

At least two different binding proteins have been identified in adult human serum, namely, IGF-binding protein 53 and IGF-binding protein 1. The IGF-binding proteins have both stimulatory and inhibitory effects on IGF. Clemmons, et al., J. Clin. Invest., 77:1548 (1986) showed increased binding to fibroblast and smooth muscle cell surface receptors of IGF in complex with its binding protein. The inhibitory effects of IGF-binding protein on various IGF actions in vitro, have been shown and they include stimulation of glucose transport by adipocytes, sulfate incorporation by chondrocytes and thymidine incorporation in fibroblast (Zapf, et al., J. Clin. Invest., 63:1077 (1979)). In addition, inhibitory effects of IGF-binding proteins on growth factor mediated mitogen activity in normal cells has been shown.

In accordance with one aspect of the present invention, there is provided a novel mature polypeptide which is VIGF, as well as biologically active and diagnostically or therapeutically useful fragments, analogs and derivatives thereof.

In accordance with another aspect of the present invention, there are provided isolated nucleic acid molecules encoding human VIGF, including mRNAs, DNAs, cDNAs, genomic DNAs as well as analogs and biologically active and diagnostically or therapeutically useful fragments and derivatives thereof.

In accordance with yet a further aspect of the present invention, there is provided a process for producing such polypeptide by recombinant techniques comprising culturing recombinant prokaryotic and/or eukaryotic host cells, containing a human VIGF nucleic acid sequence, under conditions promoting expression of said protein and subsequent recovery of said protein.

In accordance with yet a further aspect of the present invention, there is provided a process of utilizing such polypeptide, or polynucleotide encoding such polypeptide for therapeutic purposes, for example, to treat muscle wasting diseases, osteoporosis, to aid in implant fixation, to stimulate wound healing or tissue regeneration, to promote angiogenesis and to proliferate vascular smooth muscle and endothelial cell production.

In accordance with yet a further aspect of the present invention, there are provided antibodies against such polypeptides.

In accordance with yet another aspect of the present invention, there are provided antagonists to such polypeptides, which may be used to inhibit the action of such polypeptides, for example, to limit the production of excess connective tissue during wound healing or pulmonary fibrosis.

In accordance with yet a further aspect of the present invention, there are also provided nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to VIGF sequences.

In accordance with still another aspect of the present invention, there are provided diagnostic assays for detecting diseases related to the under-expression and over-expression of the VIGF polypeptide and mutations in the nucleic acid sequences encoding such polypeptide.

These and other aspects of the present invention should be apparent to those skilled in the art from the teachings herein.

Brief Description of the Drawings  
The following drawings are il-

*Brief Description of the Drawing*  
The following drawings are illustrative of embodiments of the invention and are not meant to limit the scope of the invention as encompassed by the claims. (Ref ID: N0021)

*invention as encompassed by* (SEQ ID NO:1)  
*Figure 1 shows the cDNA and corresponding deduced amino acid sequence of the VIGF polypeptide. The initial 21 amino acids represent the putative leader sequence such that the mature polypeptide comprises 163 amino acids. The standard one letter abbreviations for amino acids are used. Sequencing was performed using a 373 Automated DNA sequencer (Applied Biosystems, Inc.). Sequencing accuracy is predicted to be greater than 97% accurate.* (SEQ ID NO:2)

INS 7 Figure 2 shows the amino acid sequence homology between VIGF and other proteins which are members of the CCN family. A3

In accordance with an aspect of the present invention, there is provided an isolated nucleic acid (polynucleotide) which encodes for the mature polypeptide having the deduced amino acid sequence of ~~Figure F~~ (SEQ ID NO:2) or for the mature polypeptide encoded by the cDNA of the clone deposited as ATCC Deposit No. 75874 on August 25, 1994.

115 A polynucleotide encoding a polypeptide of the present  
116 invention may be obtained from human umbilical vein and  
117 aortic endothelial cells, aortic smooth muscle cells, and  
118 pulmonary artery. The polynucleotide of this invention was  
119 discovered in a cDNA library derived from human umbilical  
120 vein endothelial cells. It is structurally related to the  
121 IBP and CCN families. It contains an open reading frame  
122 encoding a protein of 184 amino acid residues of which  
123 approximately the first 21 amino acids residues are the  
124 putative leader sequence such that the mature protein  
125 comprises 163 amino acids.

The designation of VIGF as a hybrid member of both the CCN growth factor and IBP families was based primarily through conservation of amino acid sequences. Similarity of VIGF to the CCN family is inferred because of the 40-45% similarity over the entire polypeptide, 12 of a total of 18

VIGF cysteines are conserved, and 94% identity with the IBP signature (GCGCCXXCAXXXXXXXC) which is perfectly conserved in every member of the CCN family.

The VIGF polypeptide also has significant similarity to the IBP family. In two adjacent regions, amino acids 30-44 (IBP signature) and 55-69, there is at least 80% identity to the IBP family. These regions are contained within the putative IGF binding domain of the IBPs. The human tissue and cell-type specific expression has been determined by Northern blot analysis. The 2.3-2.4 kb VIGF mRNA is localized in the adult lung and kidney as shown using the procedure of Example 4. VIGF gene expression was undetectable in heart, brain, placenta, liver, skeletal muscle, and pancreas. Cultured human umbilical vein endothelial and aortic smooth muscle cells are cell-types which express VIGF mRNA at a high level while dermal foreskin fibroblasts show a very low level. Together, these results indicate that VIGF is primarily of vascular origin.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the mature polypeptide may be identical to the coding sequence shown in Figure 1 (SEQ ID NO:1) or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same mature polypeptide as the DNA of Figure 1 (SEQ ID NO:1) or the deposited cDNA.

The polynucleotide which encodes for the mature polypeptide of <sup>Figures 10, 11, 12, 13, 14, and 15, collectively</sup> Figure 1 (SEQ ID NO:2) or for the mature polypeptide encoded by the deposited cDNA may include: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding

sequence such as a leader or secretory sequence or a proprotein sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide.

Thus, the term "polynucleotide encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide as well as a polynucleotide which includes additional coding and/or non-coding sequence.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of ~~Figure 1~~ (SEQ ID NO:2) or the polypeptide encoded by the cDNA of the deposited clone. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide.

Thus, the present invention includes polynucleotides encoding the same mature polypeptide as shown in ~~Figure 1~~ (SEQ ID NO:2) or the same mature polypeptide encoded by the cDNA of the deposited clone as well as variants of such polynucleotides which variants encode for a fragment derivative or analog of the polypeptide of ~~Figure 1~~ (SEQ ID NO:2) or the polypeptide encoded by the cDNA of the deposited clone. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotide may have a coding sequence which is a naturally occurring allelic variant of the coding sequence shown in ~~Figure 1~~ (SEQ ID NO:1) or of the coding sequence of the deposited clone. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does

not substantially alter the function of the encoded polypeptide.

The present invention also includes polynucleotides, wherein the coding sequence for the mature polypeptide may be fused in the same reading frame to a polynucleotide sequence which aids in expression and secretion of a polypeptide from a host cell, for example, a leader sequence which functions as a secretory sequence for controlling transport of a polypeptide from the cell. The polypeptide having a leader sequence is a preprotein and may have the leader sequence cleaved by the host cell to form the mature form of the polypeptide. The polynucleotides may also encode for a proprotein which is the mature protein plus additional 5' amino acid residues. A mature protein having a prosequence is a proprotein and is an inactive form of the protein. Once the prosequence is cleaved an active mature protein remains.

Thus, for example, the polynucleotide of the present invention may encode for a mature protein, or for a protein having a prosequence or for a protein having both a prosequence and a presequence (leader sequence).

The polynucleotides of the present invention may also have the coding sequence fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. The marker sequence may be a hexahistidine tag supplied by a pQE-9 vector to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., et al., Cell, 37:767 (1984)).

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region (leader and trailer) as well

as intervening sequences (introns) between individual coding segments (exons).

Fragments of the full length gene of the present invention may be used as a hybridization probe for a cDNA library to isolate the full length cDNA and to isolate other cDNAs which have a high sequence similarity to the gene or similar biological activity. Probes of this type preferably have at least 30 bases and may contain, for example, 50 or more bases. The probe may also be used to identify a cDNA clone corresponding to a full length transcript and a genomic clone or clones that contain the complete gene including regulatory and promotor regions, exons, and introns. An example of a screen comprises isolating the coding region of the gene by using the known DNA sequence to synthesize an oligonucleotide probe. Labeled oligonucleotides having a sequence complementary to that of the gene of the present invention are used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The present invention further relates to polynucleotides which hybridize to the hereinabove-described sequences if there is at least 70%, preferably at least 90%, and more preferably at least 95% identity between the sequences. The present invention particularly relates to polynucleotides which hybridize under stringent conditions to the hereinabove-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences. The polynucleotides which hybridize to the hereinabove described polynucleotides in a preferred embodiment encode polypeptides which either retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNAs of Figure 1 (SEQ ID NO:1) or the deposited cDNA(s).

Alternatively, the polynucleotide may have at least 20 bases, preferably 30 bases, and more preferably at least 50 bases which hybridize to a polynucleotide of the present invention and which has an identity thereto, as hereinabove described, and which may or may not retain activity. For example, such polynucleotides may be employed as probes for the polynucleotide of SEQ ID NO:1, for example, for recovery of the polynucleotide or as a diagnostic probe or as a PCR primer.

Thus, the present invention is directed to polynucleotides having at least a 70% identity, preferably at least 90% and more preferably at least a 95% identity to a polynucleotide which encodes the polypeptide of SEQ ID NO:2 as well as fragments thereof, which fragments have at least 30 bases and preferably at least 50 bases and to polypeptides encoded by such polynucleotides.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to a VIGF polypeptide which has the deduced amino acid sequence of Figures 1A, 1B, 1C, 1D and 1E collectively Figure 1 (SEQ ID NO:2) or which has the amino acid sequence encoded by the deposited cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 1 (SEQ ID NO:2) or that encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide, preferably a recombinant polypeptide.

The fragment, derivative or analog of the polypeptide of Figures 1A, 1B, 1C, 1D and 1E, collectively, or that encoded by the deposited cDNA may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living

animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the

invention and the production of polypeptides of the invention by recombinant techniques.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the VIGF genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative

examples of such promoters, there may be mentioned: LTR or SV40 promoter, the E. coli. lac or trp, the phage lambda  $P_L$  promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as yeast; insect cells such as Drosophila S2 and Spodoptera Sf9; adenoviruses; animal cells such as CHO, COS or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors

and promoters are known to those of skill in the art, and are commercially available. The following vectors are provided by way of example. Bacterial: pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); pTRC99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). However, any other plasmid or vector may be used as long as they are replicable and viable in the host.

Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and PCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda  $P_R$ ,  $P_L$  and trp. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., Battey, J., *Basic Methods in Molecular Biology*, (1986)).

The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor, N.Y., (1989), the disclosure of which is hereby incorporated by reference.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of E. coli and S. cerevisiae TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK),  $\alpha$ -factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics,

e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw

cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell*, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

The VIGF polypeptides can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The polypeptides of the present invention may be a naturally purified product, or a product of chemical synthetic procedures, or produced by recombinant techniques from a prokaryotic or eukaryotic host (for example, by bacterial, yeast, higher plant, insect and mammalian cells in culture). Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated.

Polypeptides of the invention may also include an initial methionine amino acid residue.

This VIGF polypeptide of the present invention may be employed in wound-healing and associated therapies concerned with re-growth of tissue, such as connective tissue, skin, bone, cartilage, muscle, lung or kidney.

VIGF polypeptide may also be employed to enhance the growth of vascular smooth muscle and endothelial cells leading to the stimulation of angiogenesis. The VIGF-mediated increase in angiogenesis would be beneficial to ischemic tissues and to collateral coronary development in the heart subsequent to coronary stenosis.

VIGF polypeptide may also be employed during implant fixation to stimulate the growth of cells around the implant and therefore, facilitate its attachment to its intended site.

VIGF polypeptide may also be employed to increase IGF stability in tissues or in serum. It may also increase binding to the IGF receptor. Since IGF has been shown in vitro to enhance human marrow erythroid and granulocytic progenitor cell growth, VIGF polypeptide may also be employed to stimulate erythropoiesis or granulopoiesis.

In accordance with yet a further aspect of the present invention, there is provided a process for utilizing such polypeptides, or polynucleotides encoding such polypeptides, as a research reagent for in vitro purposes related to scientific research, synthesis of DNA and manufacture of DNA vectors, for the purpose of developing therapeutics and diagnostics for the treatment of human disease.

This invention provides a method for identification of the receptor for VIGF. The gene encoding the receptor can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting (Coligan, et al., Current Protocols in Immun., 1(2), Chapter 5, (1991)). Preferably, expression cloning is employed wherein

polyadenylated RNA is prepared from a cell responsive to VIGF, and a cDNA library created from this RNA is divided into pools and used to transfect COS cells or other cells that are not responsive to VIGF. Transfected cells which are grown on glass slides are exposed to labeled VIGF. VIGF can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis. Positive pools are identified and sub-pools are prepared and retransfected using an iterative sub-pooling and rescreening process, eventually yielding a single clone that encodes the putative receptor.

As an alternative approach for receptor identification, labeled VIGF can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-ray film. The labeled complex containing the VIGF-receptor can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

This invention is also related to a method of screening compounds to identify those which mimic VIGF (agonists) or prevent the effect of VIGF. An example of such a method takes advantage of the ability of VIGF to stimulate the proliferation of endothelial cells in the presence of the comitogen Con A. Human umbilical vein endothelial cells are obtained and cultured in 96-well flat-bottomed culture plates (Costar, Cambridge, MA) and supplemented with a reaction mixture appropriate for facilitating proliferation of the cells, the mixture containing Con-A (Calbiochem, La Jolla, CA). Con-A and the compound to be screened are added and after incubation at 37°C, cultures are pulsed with

3[H]thymidine and harvested onto glass fiber filters (PhD; Cambridge Technology, Watertown, MA). Mean 3[H]-thymidine incorporation (cpm) of triplicate cultures is determined using a liquid scintillation counter (Beckman Instruments, Irvine, CA). Significant 3[H]-thymidine incorporation indicates stimulation of endothelial cell proliferation.

To assay for antagonists, the assay described above is performed, however, in this assay VIGF is added along with the compound to be screened and the ability of the compound to inhibit 3[H]-thymidine incorporation in the presence of VIGF, indicates that the compound is an antagonist to VIGF. Alternatively, VIGF antagonists may be detected by combining VIGF and a potential antagonist with membrane-bound VIGF receptors or recombinant receptors under appropriate conditions for a competitive inhibition assay. VIGF can be labeled, such as by radioactivity, such that the number of VIGF molecules bound to the receptor can determine the effectiveness of the potential antagonist.

Also, a mammalian cell or membrane preparation expressing the VIGF receptor would be incubated with labeled VIGF in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured. Alternatively, VIGF, labelled IGF and a potential compound could be incubated under conditions where VIGF would naturally bind to IGF. The extent of this interaction could be measured to determine if the compound is an effective antagonist or agonist.

Examples of potential VIGF antagonists include an antibody, or in some cases, an oligonucleotide, which binds to the polypeptide. Alternatively, a potential antagonist may be a closely related protein, for example, a mutated form of VIGF, which recognizes the VIGF receptor but imparts no effect, thereby competitively inhibiting the action of VIGF.

Another potential VIGF antagonist is an antisense construct prepared using antisense technology. Antisense

technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes for the mature polypeptides of the present invention, is used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple helix -see Lee et al., Nucl. Acids Res., 6:3073 (1979); Cooney et al, Science, 241:456 (1988); and Dervan et al., Science, 251: 1360 (1991)), thereby preventing transcription and the production of VIGF. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the VIGF (antisense - Okano, J. Neurochem., 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of VIGF.

Potential VIGF antagonists include small molecules which bind to the active site, the receptor binding site, IGF or other growth factor binding site of the polypeptide thereby blocking the normal biological activity of VIGF. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

The antagonists may be employed to inhibit tumor neovascularization and the neointimal proliferation of smooth muscle cells prevalent in atherosclerosis and restenosis subsequent to balloon angioplasty.

The antagonists may also be employed to inhibit the over production of scar tissue seen in a keloid which forms after surgery, fibrosis after myocardial infarction, or fibrotic lesions associated with pulmonary fibrosis. The antagonists

may be employed in a composition with a pharmaceutically acceptable carrier, e.g., as hereinafter described.

The VIGF polypeptides and antagonist or agonists of the present invention may be employed in combination with a suitable pharmaceutical carrier. Such compositions comprise a therapeutically effective amount of the polypeptide, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration. In addition, the pharmaceutical compositions may be employed in conjunction with other therapeutic compounds.

The pharmaceutical compositions may be administered in a convenient manner such as by the oral, topical, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes. The pharmaceutical compositions are administered in an amount which is effective for treating and/or prophylaxis of the specific indication. In general, they are administered in an amount of at least about 10  $\mu$ g/kg body weight and in most cases they will be administered in an amount not in excess of about 8 mg/Kg body weight per day. In most cases, the dosage is from about 10  $\mu$ g/kg to about 1 mg/kg body weight daily, taking into account the routes of administration, symptoms, etc.

VIGF in combination with other growth factors including but not limited to, PDGF, IGF, FGF, EGF or TGF- $\beta$  may accelerate physiological responses as seen in wound healing.

The VIGF polypeptide and agonists and antagonists which are polypeptides, may also be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by, for example, procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

Retroviruses from which the retroviral plasmid vectors hereinabove mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus,

Myeloproliferative Sarcoma Virus, and ~~l~~ mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

The vector includes one or more promoters. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., Biotechniques, Vol. 7, No. 9, 980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and  $\beta$ -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention is under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter; the ApoAI promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs hereinabove described); the  $\beta$ -actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317,  $\psi$ -2,  $\psi$ -AM, PA12, T19-14X,

VT-19-17-H2,  $\psi$ CRE,  $\psi$ CRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy, Vol. 1, pgs. 5-14 (1990), which is incorporated herein by reference in its entirety. The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and  $\text{CaPO}_4$  precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either *in vitro* or *in vivo*. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

This invention is also related to the use of the VIGF gene as a diagnostic. Detection of a mutated form of VIGF will allow a diagnosis of a disease or a susceptibility to a disease, such as a tumor, since mutations in VIGF may cause tumors.

Individuals carrying mutations in the human VIGF gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki et al., *Nature*, 324:163-166 (1986)) prior to analysis. RNA or cDNA may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid

encoding VIGF can be used to identify and analyze VIGF mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled VIGF RNA or alternatively, radiolabeled VIGF antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers et al., *Science*, 230:1242 (1985)).

Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton et al., *PNAS, USA*, 85:4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., *Restriction Fragment Length Polymorphisms (RFLP)*) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations can also be detected by *in situ* analysis.

VIGF protein expression may be linked to vascular disease or neovascularization associated with tumor formation. VIGF has a signal peptide and the mRNA is highly

expressed in endothelial cells and to a lesser extent in smooth muscle cells which indicates that the protein is present in serum. Accordingly, an anti-VIGF antibody could be used to diagnose vascular disease or neovascularization associated with tumor formation since an altered level of this polypeptide may be indicative of such disorders.

A competition assay may be employed wherein antibodies specific to VIGF is attached to a solid support and labeled VIGF and a sample derived from the host are passed over the solid support and the amount of label detected attached to the solid support can be correlated to a quantity of VIGF in the sample.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

Briefly, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide

primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization (FISH) of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60 bases. For a review of this technique, see Verma et al., *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man* (available on line through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, *Nature*, 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, *Immunology Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole, et al., 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The present invention will be further described with reference to the following examples; however, it is to be understood that the present invention is not limited to such examples. All parts or amounts, unless otherwise specified, are by weight.

In order to facilitate understanding of the following examples certain frequently occurring methods and/or terms will be described.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids in accord with published procedures. In addition, equivalent plasmids to those described are known in the art and will be apparent to the ordinarily skilled artisan.

"Digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements were used as would be known to the ordinarily skilled artisan. For analytical purposes, typically 1  $\mu$ g of plasmid or DNA fragment is used with about 2 units of enzyme in about 20  $\mu$ l of buffer solution. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50  $\mu$ g of DNA are digested with 20 to 250 units of enzyme in a larger volume. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation times of about 1 hour at 37°C are ordinarily used, but may vary in accordance with the supplier's instructions. After digestion the reaction is electrophoresed directly on a polyacrylamide gel to isolate the desired fragment.

Size separation of the cleaved fragments is performed using 8 percent polyacrylamide gel described by Goeddel, D. et al., Nucleic Acids Res., 8:4057 (1980).

"Oligonucleotides" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Such synthetic oligonucleotides have no 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase. A synthetic oligonucleotide will ligate to a fragment that has not been dephosphorylated.

"Ligation" refers to the process of forming phosphodiester bonds between two double stranded nucleic acid fragments (Maniatis, T., et al., Id., p. 146). Unless otherwise provided, ligation may be accomplished using known buffers and conditions with 10 units of T4 DNA ligase ("ligase") per 0.5  $\mu$ g of approximately equimolar amounts of the DNA fragments to be ligated.

Unless otherwise stated, transformation was performed as described in the method of Graham, F. and Van der Eb, A., Virology, 52:456-457 (1973).

#### Example 1

##### Bacterial Expression and Purification of VIGF

The DNA sequence encoding VIGF, ATCC # 75874, was initially amplified using PCR oligonucleotide primers corresponding to the 5' sequences of the processed VIGF protein (minus the signal peptide sequence) and the vector sequences 3' to the VIGF gene. Additional nucleotides corresponding to VIGF were added to the 5' and 3' sequences respectively. The 5' oligonucleotide primer has the sequence 5' **CGCAAGCTTAAATAATTATGCGGTGGACTGC** 3' (SEQ ID NO:3) contains a Hind III restriction enzyme site (in bold) followed by 21 nucleotides of VIGF coding sequence starting from the presumed terminal amino acid of the processed protein codon (underlined). The 3' oligonucleotide primer 5' (underlined).

CGCTCTAGATCAGCGTGGATTTAACCA 3' (SEQ ID NO:4) contains an Xba I restriction site (in bold) followed by the reverse complement of nucleotides corresponding to the carboxy-terminal 5 amino acids and the translational stop codon (underlined). The restriction enzyme sites correspond to the restriction enzyme sites on the bacterial expression vector pQE-9 (Qiagen, Inc. Chatsworth, CA.). pQE-9 encodes antibiotic resistance (Amp'), a bacterial origin of replication (ori), an IPTG-regulatable promoter operator (P/O), a ribosome binding site (RBS), a 6-His tag and restriction enzyme sites. The VIGF PCR product and pQE-9 were then digested with Hind III and Xba I and ligated together with T4 DNA ligase. The desired recombinants would contain the VIGF coding sequence inserted downstream from the pQE-9 encoded histidine tag and the ribosome binding site. The ligation mixture was then used to transform E. coli strain M15[pREP4] (Qiagen, Inc.) by the procedure described in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Laboratory Press, (1989). M15[pREP4] contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan'). Transformants were identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies were selected. Plasmid DNA was isolated and confirmed by restriction analysis. Clones containing the desired constructs were grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture was used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells were grown to an optical density 600 (O.D. <sup>600</sup>) of between 0.4 and 0.6. IPTG ("Isopropyl-B-D-thiogalacto pyranoside") was then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression. Cells were grown an extra 3 to 4 hours such that there is an exponential growth culture

present. Cells were then harvested by centrifugation. The VIGF/6-Histidine-containing M15 [pREP4] cells were lysed in 6M GnHCl, 50 mM NaPO<sub>4</sub> at pH 8.0. The lysate was loaded on a Nickel-Chelate column and the flow-through collected. The column was washed with 6M GnHCl, 50 mM NaPO<sub>4</sub> at pH 8.0, 6.0 and 5.0. The VIGF fusion protein (>90% pure) was eluted at pH 2.0. For the purpose of renaturation, the pH 2.0 eluate was adjusted to 3 molar guanidine HCl, 100mM sodium phosphate, 10 mmolar glutathione (reduced) and 2 mmolar glutathione (oxidized). After incubation in this solution for 12 hours the protein was dialyzed to 10 mmolar sodium phosphate. To run the gel, the pellets were resuspended in SDS/NaOH and SDS-PAGE loading buffer, heat denatured, then electrophoresed on a 15% denaturing polyacrylamide gel. The Gibco BRL low range molecular weight standard was also electrophoresed (lane 1). The proteins were visualized with Coomassie Brilliant Blue R-250 stain.

#### Example 2

#### Cloning and expression of VIGF using the baculovirus expression system

The DNA sequence encoding the full length VIGF protein, ATCC # 75874, is digested with the restriction enzymes PvuII and XbaI. The 639 nucleotide PvuII, XbaI fragment contains the entire VIGF coding region plus 11 and 77 nucleotides of 5' and 3' untranslated DNA, respectively. This fragment, designated F2, is isolated from a 1% agarose gel using a commercially available kit ("Geneclean", BIO 101 Inc., La Jolla, Ca.).

The vector pA2 is used for the expression of the VIGF protein using the baculovirus expression system (for review see: Summers, M.D. and Smith, G.E. 1987, A manual of methods for baculovirus vectors and insect cell culture procedures, Texas Agricultural Experimental Station Bulletin No. 1555). This expression vector contains the strong polyhedrin

promoter of the *Autographa californica* nuclear polyhidrosis virus (AcMNPV) followed by the recognition sites for the restriction endonucleases SmaI and XbaI. The polyadenylation site of the simian virus (SV)40 is used for efficient polyadenylation. For an easy selection of recombinant viruses the beta-galactosidase gene from *E.coli* is inserted in the same orientation as the polyhedrin promoter followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for the cell-mediated homologous recombination of cotransfected wild-type viral DNA. Many other baculovirus vectors could be used in place of pA2 such as, pRG1, pAc373, pVL941 and pAcIM1 (Luckow, V.A. and Summers, M.D., *Virology*, 170:31-39).

The plasmid is digested with the restriction enzymes SmaI and XbaI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The DNA is then isolated from a 1% agarose gel using the commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated V2.

Fragment F2 and the dephosphorylated plasmid V2 are ligated with T4 DNA ligase. *E.coli* strain XL1 Blue (Stratagene Cloning Systems, 11011 North Torrey Pines Road La Jolla, Ca. 92037) are then transformed and bacteria identified that contained the plasmid (pBac VIGF) with the VIGF cDNA using the enzymes BamHI and XbaI. The sequence of the cloned fragment is confirmed by DNA sequencing.

5  $\mu$ g of the plasmid pBac VIGF is cotransfected with 1.0  $\mu$ g of a commercially available linearized baculovirus ("BaculoGold" baculovirus DNA", Pharmingen, San Diego, CA.) using the lipofection method (Felgner et al. *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987)).

1 $\mu$ g of BaculoGold<sup>TM</sup> virus DNA and 5  $\mu$ g of the plasmid pBac VIGF are mixed in a sterile well of a microtiter plate containing 50  $\mu$ l of serum free Grace's medium (Life

Technologies Inc., Gaithersburg, MD). Afterwards 10  $\mu$ l Lipofectin plus 90  $\mu$ l Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added dropwise to the Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1ml Grace' medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation continued at 27°C for four days.

After four days the supernatant is collected and a plaque assay performed similar as described by Summers and Smith (supra). As a modification an agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used which allows an easy isolation of blue stained plaques. (A detailed description of a "plaque assay" can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after the serial dilution, the viruses are added to the cells and blue stained plaques are picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses is then resuspended in an Eppendorf tube containing 200  $\mu$ l of Grace's medium. The agar is removed by a brief centrifugation and the supernatant containing the recombinant baculoviruses is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then stored at 4°C.

Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V-VIGF at a multiplicity of infection (MOI) of 2. Six hours later the medium is removed and replaced with SF900 II medium minus methionine and cysteine

(Life Technologies Inc., Gaithersburg). 42 hours later 5  $\mu$ Ci of  $^{35}$ S-methionine and 5  $\mu$ Ci  $^{35}$ S cysteine (Amersham) are added. The cells are further incubated for 16 hours before they are harvested by centrifugation and the labelled proteins visualized by SDS-PAGE and autoradiography.

### Example 3

#### Expression of Recombinant VIGF in CHO cells

The vector pN346 is used for the expression of the VIGF protein. Plasmid pN346 is a derivative of the plasmid pSV2-dhfr [ATCC Accession No. 37146]. Both plasmids contain the mouse dhfr gene under control of the SV40 early promoter. Chinese hamster ovary or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F.W., Kellems, R.M., Bertino, J.R., and Schimke, R.T., 1978, J. Biol. Chem. 253:1357-1370, Hamlin, J.L. and Ma, C. 1990, Biochem. et Biophys. Acta, 1097:107-143, Page, M.J. and Sydenham, M.A. 1991, Biotechnology Vol. 9:64-68). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the dhfr gene it is usually co-amplified and overexpressed. Subsequently, when the methotrexate is withdrawn, cell lines contain the amplified gene integrated into the chromosome(s).

Plasmid pN346 contains for the expression of the gene of interest a strong promoter of the long terminal repeat (LTR) of the Rous Sarcoma Virus (Cullen, et al., Molecular and Cellular Biology, March 1985, 438-447) plus a fragment isolated from the enhancer of the immediate early gene of

human cytomegalovirus (CMV) (Boshart et al., Cell 41:521-530, 1985). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: BamHI, Pvull, and Nrul. Behind these cloning sites the plasmid contains translational stop codons in all three reading frames followed by the 3' intron and the polyadenylation site of the rat preproinsulin gene. Other high efficient promoters can also be used for the expression, e.g., the human  $\beta$ -actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLV. For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well.

Stable cell lines carrying a gene of interest integrated into the chromosome can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g. G418 plus methotrexate.

The plasmid pN346 is digested with the restriction enzyme BamHI and then dephosphorylated using calf intestinal phosphatase by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the full length VIGF protein, ATCC #75874, is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence 5' CGCAGATCTCCGCCACCTGAA GAGCGTCTTGCTGCTG 3' (SEQ ID NO:5) and contains a BglII restriction enzyme site (in bold) followed by 8 nucleotides resembling an efficient signal for the initiation of translation in eukaryotic cells (Kozak, M., J. Mol. Biol., 196:947-950, (1987)). The remaining nucleotides correspond to the amino terminal 7 amino acids including the translational initiation codon (underlined). The 3' primer has the sequence 5' CGCAGATCTAGCCTCTCAGAAATCACA 3' (SEQ ID NO:6) and contains a BglII restriction site (in bold) and 21

nucleotides that are the reverse complement of 3' untranslated DNA starting 7 nucleotides downstream from the translational stop codon. The PCR product is digested with BglII and purified on a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). This fragment is then ligated to BamHI digested, phosphatased pN346 plasmid with T4 DNA ligase. X11Blue (Stratagene) E. coli are transformed and plated on LB, 50  $\mu$ g/ml ampicillin plates. Colonies bearing the desired recombinant in the proper orientation are screened for by PCR with a 5' primer which corresponds to the Rous sarcoma virus promoter and a 3' primer which corresponds to the reverse complement of VIGF codons 73-79. The sequence of the cloned fragment is confirmed by DNA sequencing. Transfection of CHO-dhfr-cells

Chinese hamster ovary cells lacking an active DHFR enzyme are used for transfection. 5  $\mu$ g of the expression plasmid pN346VIGF are cotransfected with 0.5  $\mu$ g of the plasmid pSVneo using the lipofectin method (Felgner et al., supra). The plasmid pSV2-neo contains a dominant selectable marker, the gene neo from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) and cultivated from 10-14 days. After this period, single clones are trypsinized and then seeded in 6-well petri dishes using different concentrations of methotrexate (25, 50 nM, 100 nM, 200 nM, 400 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (500 nM, 1  $\mu$ M, 2  $\mu$ M, 5  $\mu$ M). The same procedure is repeated until clones grew at a concentration of 100  $\mu$ M.

The expression of the desired gene product is analyzed by Western blot analysis and SDS-PAGE.

Example 4

Tissue Localization of VIGF Gene Expression by Northern Blot Analysis

A multiple tissue Northern blot (Clontech Laboratories, Inc., 4030 Fabian Way, Palo Alto, California 94303) containing 2 ug of human adult brain, heart, placenta, lung, liver skeletal muscle, kidney, and pancreas poly A+ mRNA per lane is prehybridized in Church buffer (Church, G. M. & Gilbert, W., Proc. Natl. Acad. Sci. USA 81, 1991-1995 (1984)) at 60°C for one hour. The DNA sequence coding for VIGF, ATCC# 75874, is amplified from the full length cDNA cloned in pBluescript SK(-) using the M13 Forward (5' GGGTTTTCCCAGTCACGAC 3') (SEQ ID NO:7) and Reverse (5' ATGCTTCCGGCTCGTATG 3') (SEQ ID NO:8) primers. Twenty-five nanograms of PCR product is random primer radiolabeled (Prime-It II, Stratagene Cloning Systems, 11011 North Torrey Pines Rd.; La Jolla, California 92037) with <sup>32</sup>P-dCTP. The heat denatured VIGF probe is added directly to the prehybridization buffer and incubated 16 hr at 60°C. Two ten minute washes are performed in 0.2X SSC, 0.1% SDS at 60°C. Autoradiography is performed at -80°C.

A 2.3 kb transcript is seen in lung and kidney after a four day exposure.

Example 5

Cell-Type Analysis of VIGF Gene Expression by Northern Blot Analysis

Human umbilical vein endothelial, aortic smooth muscle, dermal foreskin fibroblast cells (Clonetics, 9620 Chesapeake Drive, Suite #201; San Diego, California 92123) were grown to 75-90% confluency. Total RNA is extracted with RNAzol (Biotecx Laboratories, Inc., 6023 South Loop East Houston, Texas 77033). A 1.2% agarose formaldehyde gel is prepared

and run with 20 ug of total RNA per lane and an RNA ladder size marker (Life Technologies, Inc., 8400 Helgerman Ct., P.O. Box 6009 Gaithersburg, Maryland 20884) according to Sambrook et al. (1989). The RNA is transferred overnight to Hybond N+ (Amersham Corp., 2636 South Clearbrook Drive; Arlington Heights, Illinois 60005) and bound to the membrane with a Stratalinker UV Crosslinker (Stratagene Cloning Systems, La Jolla, California). The blot is prehybridized in Church buffer (Church, G. M. & Gilbert, W., PNAS, USA 81:1991-1995 (1984)) at 60°C for one hour. The DNA sequence encoding VIGF, ATCC # 75874, is amplified from the full length cDNA cloned in pBluescript SK(-) using the M13 Forward (5' GGGTTTCCCCAGTCACGAC 3') (SEQ ID NO:9) and Reverse (5' ATGCTTCCGGCTCGTATG 3') (SEQ ID NO:10) primers. Twenty-five nanograms of PCR product is random primer radiolabeled (Prime-It II, Stratagene) with <sup>32</sup>P-dCTP. The heat denatured VIGF probe is added directly to the prehybridization buffer and incubated 16 hr at 60°C. Two ten minute washes were performed in 0.2X SSC, 0.1% SDS at 60°C. Autoradiography is performed at -80°C. A 2.3-2.4 kb transcript is seen in umbilical vein endothelial (lane 1) and aortic smooth muscle cells (lane 2) after a two hour exposure and also in dermal foreskin fibroblast (lane 3) cells after a 36 hour exposure.

### Example 6

## Expression via Gene Therapy

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12

media, with 10% FBS, penicillin and streptomycin, is added. This is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al, DNA, 7:219-25 (1988) flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

The cDNA encoding a polypeptide of the present invention is amplified using PCR primers which correspond to the 5' and 3' end sequences respectively. The 5' primer containing an EcoRI site and the 3' primer further includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is used to transform bacteria HB101, which are then plated onto agar-containing kanamycin for the purpose of confirming that the vector had the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells are transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is harvested from a 10 cm plate of confluent producer cells. The spent media, containing the

infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his.

The engineered fibroblasts are then injected into the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads. The fibroblasts now produce the protein product.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: HASTINGS, ET AL.

(ii) TITLE OF INVENTION: Human Vascular IBP-Like Growth Factor

(iii) NUMBER OF SEQUENCES: 17

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: CARELLA, BYRNE, BAIN, GILFILLAN,  
CECCHI, STEWART & OLSTEIN

(B) STREET: 6 BECKER FARM ROAD

(C) CITY: ROSELAND

(D) STATE: NEW JERSEY

(E) COUNTRY: USA

(F) ZIP: 07068

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 INCH DISKETTE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: MS-DOS

(D) SOFTWARE: WORD PERFECT 5.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 08/464,339

(B) FILING DATE: June 5, 1995

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA

(A) APPLICATION NUMBER: PCT/US94/14388

(B) FILING DATE: 9 DEC 1994

(viii) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: 325800-332

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 201-994-1700

(B) TELEFAX: 201-994-1744

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 1271 BASE PAIRS

(B) TYPE: NUCLEIC ACID

(C) STRANDEDNESS: SINGLE

(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCTTCCCA	CCAGCAAAGA	CCACGACTGG	AGAGCCGAGC	CGGAGCAGCT	GGGAAACATG	60
AAGAGCGTCT	TGCTGCTGAC	CACGCTCCTC	GTGCCTGCAC	ACCTGGTGGC	CGCCTGGAGC	120
AATAATTATG	CGGTGGACTG	CCCTCAACAC	TGTGACAGCA	GTGAGTGCAA	AAGCAGCCCG	180
CGCTGCAAGA	GGACAGTGT	CGACGACTGT	GGCTGCTGCC	GAGTGTGC	TGCAGGGCGG	240
GGAGAAACTT	GCTACCGCAC	AGTCTCAGGC	ATGGATGGCA	TGAAGTGTGG	CCCGGGGCTG	300
AGGTGTCAGC	CTTCTAATGG	GGAGGATCCT	TTTGGTGAAG	AGTTGGTAT	CTGCAAAGAC	360
TGTCCCTACG	GCACCTTCGG	GATGGATTGC	AGAGAGACCT	GCAACTGCCA	GTCAGGCATC	420
TGTGACAGGG	GGACGGGAAA	ATGCCTGAAA	TTCCCTTCT	TCCAATATT	AGTAACCAAG	480
TCTTCCAACA	GATTGTTTC	TCTCACGGAG	CATGACATGG	CATCTGGAGA	TGGCAATATT	540
GTGAGAGAAG	AAGTTGTGAA	AGAGAATGCT	GCCGGGTCTC	CCGTAATGAG	GAAATGGTTA	600
AATCCACGCT	GATCCCGGCT	GTGATTCTG	AGAGAAGGCT	CTATTTCGT	GAYTGTCAA	660
CACACAGCCA	ACATTTAGG	AACTTTCTAG	ATTATAGCAT	AAGGACATGT	AATTTTTGAA	720
GACCAAATGT	GATGCATGGT	GGATCCAGAA	AACAAAAAGT	AGGATACTTA	CAATCCATAA	780
CATCCATATG	ACTGAACACT	TGTATGTGTT	TGTTAAATAT	TCGAATGCAT	GTAGATTTGT	840
TAAATGTGTG	TGTATAGTAA	CACTGAAGAA	CTAAAAATGC	AATTTAGGTA	ATCTTACATG	900
GAGACAGGTC	AACCAAAGAG	GGAGCTAGGC	AAAGCTGAAG	ACCGCAGTGA	GTCAAATTAG	960
TTCTTGACT	TTGATGTACA	TTAATGTTGG	GATATGGAAT	GAAGACTTAA	GAGCAGGAGA	1020
AGATGGGGAG	GGGGTGGGAG	GGGGAAATAA	AATATTTAGC	CCTTCCTTGG	TAGGTAGCTT	1080
CTCTAGAATT	TAATTRTGCT	TTTTTTTTTT	TTTTTGGGCT	TTGGGAAAAG	TCAAAATAAA	1140
ACAACCAGAA	AACCCCTGAA	GGAAAGTAAGA	TGTTTGAAGC	TTATGAAAT	TTGAGTAACA	1200
AACAGCTTG	ANCTGAGAGC	AATTYCAAAA	GGCTGCTGAT	GTAGCCCCCG	GGTTNCCTNT	1260
NTCTNAAGGA	C					1271

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 184 AMINO ACIDS
- (B) TYPE: AMINO ACID
- (C) STRANDEDNESS:
- (D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Lys	Ser	Val	Leu	Leu	Leu	Thr	Thr	Leu	Leu	Val	Pro	Ala	His
							-20		-15					-10
Leu	Val	Ala	Ala	Trp	Ser	Asn	Asn	Tyr	Ala	Val	Asp	Cys	Pro	Gln
									1			5		
His	Cys	Asp	Ser	Ser	Glu	Cys	Lys	Ser	Ser	Pro	Arg	Cys	Lys	Arg
							10		15		20			
Thr	Val	Leu	Asp	Asp	Cys	Gly	Cys	Cys	Arg	Val	Cys	Ala	Ala	Gly
							25		30		35			
Arg	Gly	Glu	Thr	Cys	Tyr	Arg	Thr	Val	Ser	Gly	Met	Asp	Gly	Met
							40		45		50			
Lys	Cys	Gly	Pro	Gly	Leu	Arg	Cys	Gln	Pro	Ser	Asn	Gly	Glu	Asp
							55		60		65			
Pro	Phe	Gly	Glu	Glu	Phe	Gly	Ile	Cys	Lys	Asp	Cys	Pro	Tyr	Gly
							70		75		80			
Thr	Phe	Gly	Met	Asp	Cys	Arg	Glu	Thr	Cys	Asn	Cys	Gln	Ser	Gly
							85		90		95			
Ile	Cys	Asp	Arg	Gly	Thr	Gly	Lys	Cys	Leu	Lys	Phe	Pro	Phe	Phe
							100		105		110			

Gln Tyr Ser Val Thr Lys Ser Ser Asn Arg Phe Val Ser Leu Thr  
115 120 125  
Glu His Asp Met Ala Ser Gly Asp Gly Asn Ile Val Arg Glu Glu  
130 135 140  
Val Val Lys Glu Asn Ala Ala Gly Ser Pro Val Met Arg Lys Trp  
145 150 155  
Leu Asn Pro Arg  
160

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 31 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGCAAGCTTA AATAATTATG CGGTGGACTG C

31

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 27 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGCTCTAGAT CAGCGTGGAT TTAACCA

27

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 38 BASE PAIRS
  - (B) TYPE: NUCLEIC ACID
  - (C) STRANDEDNESS: SINGLE
  - (D) TOPOLOGY: LINEAR
- (ii) MOLECULE TYPE: Oligonucleotide
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCAGATCTC CGCCACCATG AAGAGCGTCT TGCTGCTG

38

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 30 BASE PAIRS

(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR  
(ii) MOLECULE TYPE: Oligonucleotide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CGCAGATCTA GCCTTCTCTC AGAAATCACA

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 19 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR  
(ii) MOLECULE TYPE: Oligonucleotide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGTTTTCCC AGTCACGAC

19

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 18 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR  
(ii) MOLECULE TYPE: Oligonucleotide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGCTTCCGG CTCGTATG

18

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 19 BASE PAIRS  
(B) TYPE: NUCLEIC ACID  
(C) STRANDEDNESS: SINGLE  
(D) TOPOLOGY: LINEAR  
(ii) MOLECULE TYPE: Oligonucleotide  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGTTTTCCC AGTCACGAC

19

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 18 BASE PAIRS

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 54 AMINO ACIDS  
(B) TYPE: AMINO ACID  
(C) STRANDEDNESS:  
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Met Glu Thr Gly Gly Gln Gln Leu Pro Val Leu Leu Leu Leu  
5 10 15  
Leu Leu Leu Leu Arg Pro Cys Glu Val Ser Gly Arg Glu Ala Ala  
20 25 30  
Cys Pro Arg Pro Cys Gly Gly Arg Cys Pro Ala Glu Pro Pro Arg  
35 40 45  
Asp Pro Met Ser Ser Glu Ala Lys Ile  
50

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 50 AMINO ACIDS  
(B) TYPE: AMINO ACID  
(C) STRANDEDNESS:  
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Gln Arg Ala Arg Pro Thr Leu Trp Ala Ala Ala Leu Thr Leu  
5 10 15  
Leu Val Leu Leu Arg Gly Pro Pro Val Ala Arg Ala Gly Ala Ser  
20 25 30  
Ser Gly Gly Leu Gly Pro Val Val Arg Cys Glu Pro Cys Val Ala  
35 40 45  
Arg Ala Leu Ala Arg  
50

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS  
(A) LENGTH: 42 AMINO ACIDS  
(B) TYPE: AMINO ACID  
(C) STRANDEDNESS:  
(D) TOPOLOGY: LINEAR

(ii) MOLECULE TYPE: PROTEIN

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Lys Ser Val Leu Leu Leu Thr Thr Leu Leu Val Pro Ala His  
5 10 15  
Leu Val Ala Ala Trp Ser Asn Met Tyr Ala Val Asp Cys Pro Gln  
20 25 30  
His Cys Asp Ser Ser Glu Cys Lys Ser Ser Pro Arg  
35 40

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